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## Full Length Research Paper

# Altered protease activity due to heat shock in the whole organism *Bombyx mori* L.

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The domesticated insect *Bombyx mori* respond well to environmental fluctuations that govern the success of cocoon production due to its poikilothermic nature. Since, the interaction between heat shock proteins and proteases in the whole organism that complete larval life in natural environment remain obscure, we have employed zymography technique to examine different classes of proteases and its activity under imposed temperature stress (heat shock) condition. The total protein extracted from the whole larvae was analyzed in SDS-polyacrylamide gel copolymerized with gelatin, casein and BSA. Discrete proteolytic bands aptly visualized in the gel contained gelatin as substrate revealed strong activity of five proteases with molecular mass of 116 (P1), 83 (P2), 57 (P3), 35 (P4) and 24 kDa (P5) and the only protease that exhibits activity in all the substrates was P5. Various protease inhibitors like leupeptine, aprotinin, pepstatin, EDTA and PMSF were used for identification and most of the proteases activity eventually was inhibited by PMSF while other inhibitors had no effect. With this we could determine the presence of large group of serine proteases in the whole body of *B. mori*. Further, for the first time, we have employed zymographic technique to evaluate protease activity in the heat shock (40°C) induced larvae. Interestingly, the activity of three proteases (P3 to P5) found inhibited on gelatin substrate while high molecular weight proteases (P1 and P2) were not affected. The protease assay further confirmed decreased protease activity in the heat shock induced larvae compared to control. The differential and altered proteases activities in normal and heat-shocked larvae respectively envisaged its significance in stress physiology and adaptation of whole organism to elevated temperature.

**Key words:** *Bombyx mori*, heat shock, lepidoptera, serine-protease, zymography.

## INTRODUCTION

Silkworm, *Bombyx mori* is the only insect that is truly domesticated and well exploited commercially for the production of silk. In recent years, it is considered as central model insect among lepidopterans (Goldsmith et al., 2004) for comparative proteome and genome studies due to its rich genetic resources. And also used as a potential tool for expression of foreign genes of interest in synthesis of recombinant proteins of pharmaceutical

importance (Tomita et al., 2003). Consequently, using advanced proteome tools and techniques protein map has been established from silkglands (Jin et al., 2004; Zhang et al., 2006), midgut (Kajiwarra et al., 2005) and whole egg (Manjunatha et al., 2005) of *B. mori*. All the information's are stored in the proteome database KAIKO-2D and accessible through <http://kaiko2ddb.dna.affrc.go.jp/>. The advent of integrated approach following electrophoretic [single dimensional electrophoresis (1-DE) and two dimensional electrophoresis (2-DE)] techniques, liquid chromatography-mass spectrometry and application of bioinformatics tools could unravel the structure and

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functional properties of biomolecules. Hence, the concept of whole organism has been followed in analysis of differential expression of heat shock proteins (HSPs) (Vasudha et al., 2006; Kundapur et al., 2009; Aparna et al., 2010) in *B. mori* and finding its evolutionary significance in post-genomic era (Sorensen and Loeschcke, 2007). Hitherto, temperature and pH known to govern the catalytic activity of an enzyme either in cells or tissues but their activity and its correlation during the period of heat shock and/or any stress conditions in the whole organism remain enigmatic.

Briefly, although heat shock response and expression of HSPs in *B. mori* have been investigated over two decades (Lohmann and Riddiford, 1992; Joy and Gopinathan, 1995; Evgen'ev et al., 2005), their biological significance and role in acquired thermotolerance remain unclear (Manjunatha et al., 2010). However, HSPs involvement in physiological and cytoprotective function (Kregel, 2002) thermotolerance in mammalian cells (Landry et al., 1989), *Drosophila* (Landry and Huot, 1995), *Musca domestica* (Tiware et al., 1997), *Lucilia cuprina* (Tiware et al., 1995), apoptosis (Arrigo, 1998, 2005), cell growth and differentiation (Mehlen et al., 1997) is obvious. As a consequence, molecular and thermal biologist's attention aids to determine the role of HSPs in the whole organism adaptation to heat including species inhabiting in various environmental conditions (Sorensen and Loeschcke, 2007).

Obviously, the structural and functional properties of proteins, which are significant in cell function and protection, are governed by different classes of proteases and analyzed employing a simple and sensitive technique "Zymography". Normally, many proteins are synthesized as inactive precursors known as zymogens and are subsequently converted to physiologically active forms by selective enzymatic cleavage of peptide bonds called zymogen activation (Neurath and Walsh, 1976). As SDS activates the zymogen into active protease, various proteases like matrix metallo proteases (MMPs) expressed during cancer development were identified and characterized (Tanriverdi et al., 2009; Lokeshwar et al., 1993). Interestingly, insect (lepidoptera) larval mid gut hosts a complex proteolytic environment of various proteases like trypsin, chymotrypsin, elastases, cathepsin-B like proteases, aminopeptidases and carboxypeptidases, which are responsible for protein digestion, killing bacteria and viruses (Nakazawa et al., 2004). In *B. mori*, serine proteases are known to dominate the larval midgut environment and contribute about 95% of the total digestive activity. Interestingly, silkworm egg also possesses some proteases that are involved in vitellin and yolk degradation during embryo development (Ikeda et al., 1990; Maki and Yamashita, 2000). Since most of these studies are confined to specific tissue, an interaction between protein and proteases in the whole organism that complete its larval

life in natural environmental conditions remain enigmatic. Thus, we have employed zymography technique to examine different classes of proteases present in the whole body of *B. mori* larvae and their altered activity under stress conditions due to heat shock.

## MATERIALS AND METHODS

### Experimental insect

The domesticated silkworm, *B. mori* eggs procured from Central Sericultural Germplasm Centre, Hosur, Tamil Nadu, India were incubated under optimum environmental conditions ( $25 \pm 1^\circ\text{C}$  and  $75 \pm 5\%$  relative humidity) until hatching. Hatched larvae were reared on mulberry leaves following the standard procedure until spinning of cocoons (Jolly, 1987).

### Sample preparation and electrophoresis

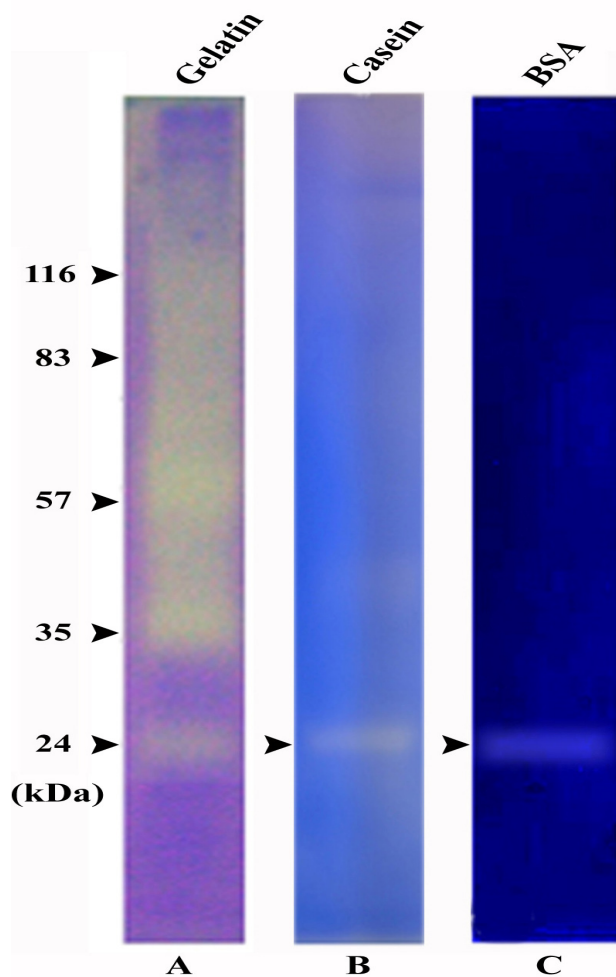
Day 3 of fifth instar silkworm larvae were given heat shock at  $40^\circ\text{C}$  for 2 h in BOD incubator with  $70 \pm 5\%$  humidity followed by 2 h recovery at room temperature. The total protein from the whole body of control and heat shocked larvae was extracted separately as described by Vasudha et al. (2006) without phenylmethanesulphonylfluoride (PMSF) and dithioerythritol/ $\beta$ -mercaptoethanol. The extracted total protein sample was incubated with various protease inhibitors like leupeptin ( $100 \mu\text{M}$ ), aprotinin ( $100 \mu\text{g/ml}$ ), pepstatin ( $10 \mu\text{M}$ ), EDTA ( $10 \text{ mM}$ ), PMSF ( $5 \text{ mM}$ ) separately for 30 min at  $4^\circ\text{C}$  prior to electrophoresis. None of these inhibitors were added to the protein sample extracted from the control and heat shocked larvae for comparative analysis. SDS-PAGE (12%) was carried out at  $4^\circ\text{C}$  incorporating different substrates, gelatin (0.1%), BSA (0.1%) and casein (1%) into the running gel, whereas only gelatin was used as substrate for the protein sample derived from heat shocked larvae.

### Zymography

The zymography was performed as described by Heussen and Dowdle (1980) with suitable modifications. The electrophoresed gel was rinsed twice in Triton X-100 (2.5%) at room temperature. In-gel protease activity was carried out incubating the gel at  $37^\circ\text{C}$  for 18 h in reaction buffer (50 mM Tris- HCl, pH 6.8). The gel was stained with Coomassie brilliant blue R-250 in isopropanol (0.25%) followed by destaining in acetic acid (7%). The protease activity was determined based on lytic bands in blue background and images were captured using Alpha Imager UV Gel Doc System and Alpha EaseFC software for molecular weight determination.

### Protease assay

The protease assay was carried out using different substrates as described by Cupp-Enyard (2008) with necessary modifications. The proteases present in the sample extracted from both normal and heat shocked silkworm larvae digest the substrates and the resultant peptides were estimated using tyrosine as standard (Lowry et al., 1951).



**Figure 1.** SDS-zymograph of proteases derived from the whole body of *Bombyx mori*. SDS-polyacrylamide gel copolymerized with gelatin (A), casein (B) and BSA (C) as substrates. Proteolytic bands along with their respective predicted molecular weight are denoted with arrows.

## RESULTS AND DISCUSSION

### Proteases and their activity

Total protein extracted from the whole body of day 3 fifth instar larvae of *B. mori* was analyzed for protease activity by zymography considering the recent concept of whole organism to understand molecular basis underlay in cellular protection and stress physiology of living organisms (Sorensen and Loeschcke, 2007; Ulmasov et al., 1992). This approach facilitated us to detect few proteolytic bands that contrasted well in the blue background of un-degraded protein substrate in Coomassie stained polyacrylamide gel. Figure 1 explicit discrete lytic zone that represents differential proteolytic activity of different proteases on various substrates

gelatin, casein and BSA.

Comparatively, five discrete proteolytic bands were noticed in the gel copolymerized with gelatin as substrate (Figure 1A), where as only one lytic zone (band) was observed in the gels supplemented with casein (Figure 1B) and BSA (Figure 1C) as substrates (Table 1). These proteolytic zones were designated as P1-P5 (P stands for protease) for comparative analysis. The molecular weight determined for the proteolytic bands was 116 (P1), 83 (P2), 57 (P3), 35 (P4) and 24 kDa (P5) (Figure 1A). Of the five different proteases, strong activity with large proteolytic band was observed with P3, where as P5 exhibited similar activity in all the substrates used (Figure 1 and Table 1). However, 24 kDa (P5) protease activity was obvious in gelatin, casein and BSA embedded gels indicating broad specificity, while other proteases have narrow specificity with gelatin. Thus, most of the proteases detected in the protein sample of day 3 of fifth instar larvae of *B. mori*, were gelatinases rather than caseinolysases and BSA degrading proteases.

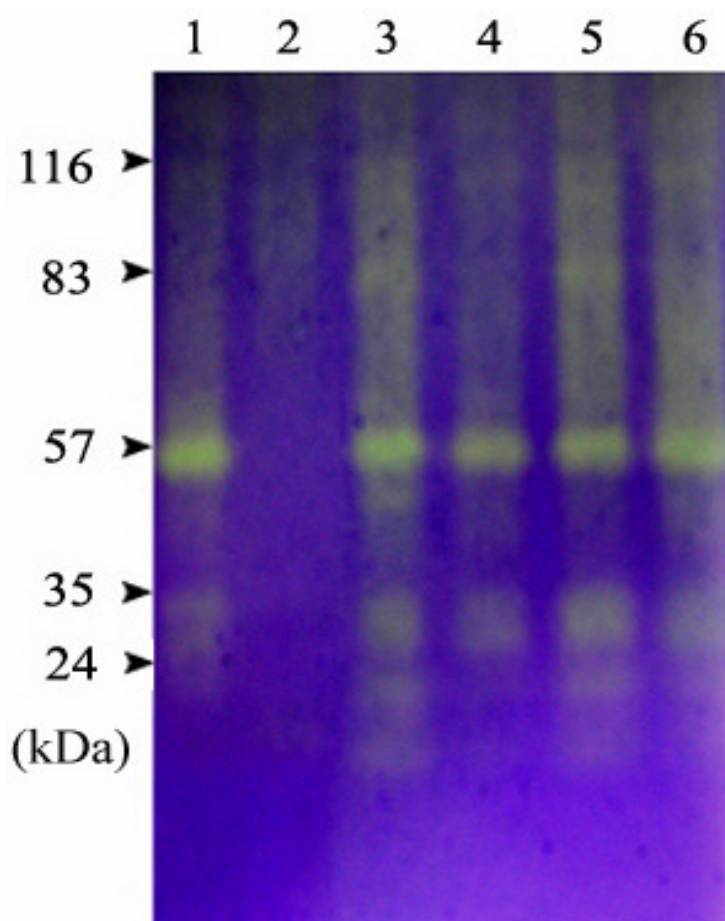
### Classification of proteases using protease inhibitors

Five different types of proteases investigated in the present study (P1 to P5) were identified and their activity was measured in presence of various protease inhibitors in the gel-containing gelatin as substrate. In the present experiment, we have incorporated various protease inhibitors (leupeptin, aprotinin, pepstatin, EDTA and PMSF) into the extraction buffer and analyzed independently. Among different protease inhibitors, PMSF inhibited the activity of all the proteases (P2 to P5) except high molecular weight protease P1 that showed partial inhibition (Figure 2 and Table 2). More interestingly, the P3 maintained its strong activity in presence of other inhibitors except in PMSF, where as P5 exhibited partial inhibition with pepstatin (Figure 2; Lane 4). Since, each well of the gel received same quantum of sample, differences in intensity and size of the lytic zones was proportional to the protease activity per sample (Naggie et al., 1997). This differential inhibition of protease activity suggested that of the five proteolytic bands (P1 to P5) are detected in the present study, three bands P2 to P4 found belong to class serine proteases as evident by their inhibition with PMSF, which is a known inhibitor of serine proteases (Figure 2; Lane 2).

Whereas, 24 kDa protease could be either serine or aspartate protease as its activity was inhibited by PMSF and pepstatin. Interestingly, a serine protease of 37 kDa was reported as zymogen from the larval midgut of *B. mori*, which is then activated after pupation. Its sequence analysis and cDNA determined the p37k as trypsin-type serine protease (Kaji et al., 2009). However, the biological significance of 24 kDa protease in fifth instar silkworm larvae is unknown, hitherto, serine proteases

**Table 1.** Differential activity of proteases on different substrates in the whole body of *Bombyx mori*.

Proteolytic bands	Mol. Wt. (kDa)	Gelatin	Casein	BSA
P1	116	+	-	-
P2	83	+	-	-
P3	57	+	-	-
P4	35	+	-	-
P5	24	+	+	+

**Figure 2.** Effect of inhibitors on proteases of *Bombyx mori*. Lane 1 - Protein sample devoid of inhibitor; Lane 2 - Protein sample with PMSF; Lane 3 - Protein sample with EDTA; Lane 4 - Protein sample with Pepstatin; Lane 5 - Protein sample with Aprotinin; Lane 6 - Protein sample with Leupeptine. All these protease inhibitors were added to extraction buffer and loaded into the gel copolymerized with gelatin as substrate.

play pivotal role in blood coagulation systems of both arthropods and mammals. More importantly, the high molecular weight protease (116 kDa – P1) detected in the whole body of *B. mori* was not inhibited by any of the inhibitors used in the present study even with increased

concentrations. This clearly indicates that this would be a new protease, which opens ample scope for molecular characterization and to determine its biological significance. Coincidentally, five different serine proteases from the haemolymph of the mosquito *Anopheles*



**Table 2.** Effect of different inhibitors on protease activity in *Bombyx mori*.

Inhibitors (concentration)	Proteolytic bands				
	P1 (116 kDa)	P2 (83 kDa)	P3 (57 kDa)	P4 (35 kDa)	P5 (24 kDa)
PMSF (5 mM)	±	+	+	+	+
EDTA (10 mM)	-	-	-	-	-
Pepstatin (10 µM)	-	-	-	-	±
Aprotinin (100 µg/ml)	-	-	-	-	-
Leupeptin (100 µM)	-	-	-	-	-

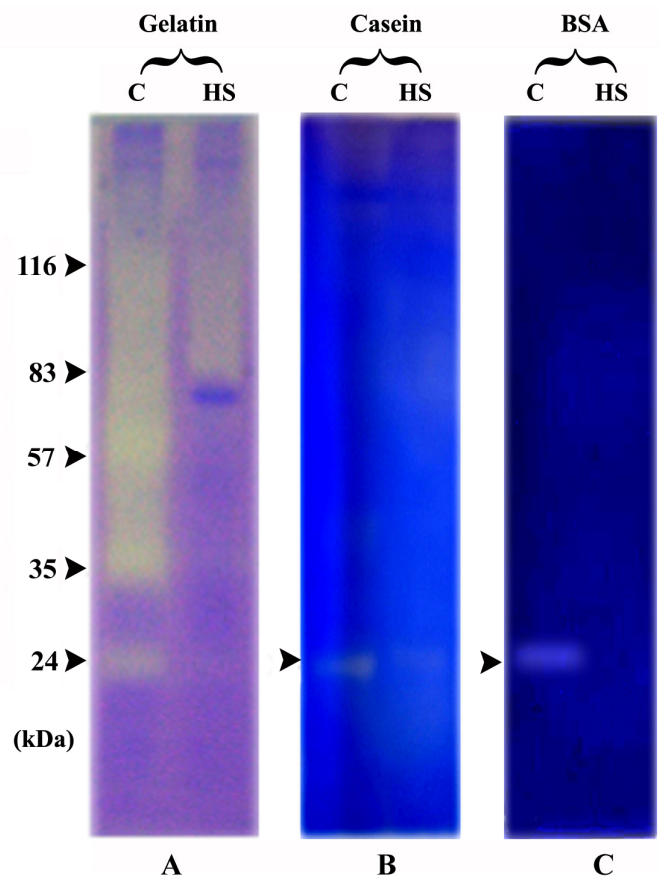
('+' Complete inhibition; '-' no inhibition; '±' partial inhibition with residual activity).

*gambiae* (Gorman and Paskewitz, 2001) and a zymogen of a serine-type protease in *B. mori* (Katsumi et al., 1995) were reported, which has an important role in insect immunity. Hence, the present data suffice an insight for detail investigation to uncover its cellular and biological significance in *B. mori*.

### Effect of heat shock on protease activity

Conventionally, zymography technique is routinely followed to identify proteolytic activity in SDS-polyacrylamide gel copolymerized with a substrate and fibrin zymography method identifies enzymes in enzyme reaction buffer containing protease inhibitors (Kim and Choi, 1998). But in the present study, we have adopted a new strategy that evaluate the protease activity as influenced by heat shock wherein the total protein extracted from the heat induced *B. mori* larvae was used without protease inhibitors either in the gel or reaction buffer.

Total proteins of the 3rd day fifth instar heat shock given and control larvae were subjected to zymography analysis devoid of protease inhibitors. Conditions maintained in zymography for heat shocked and control samples were identical to make comparative analysis more precise and accurate in different protein substrates. Interestingly, of the five active proteases (P1 to P5) extracted from the larvae without heat shock, a complete inhibition of P3, P4 and P5 and no inhibition of P1 and of P2 were observed in the protein sample derived from heat shock induced larvae (Figure 3A and Table 3). In casein and BSA embedded gels, the activity of P5 was found normal in control larvae, while a partial inhibition in its activity observed in the protein samples of heat shock induced larvae (Figures 3B, C and Table 3). Surprisingly, high molecular weight proteases P1 (116 kDa) and P2 (83 kDa) were not affected due to heat shock. During this experiment, care was taken not to boil the protein samples extracted from the heat shocked and control silkworm larvae before loading them into the gel for electrophoresis in order to substantiate the effect of



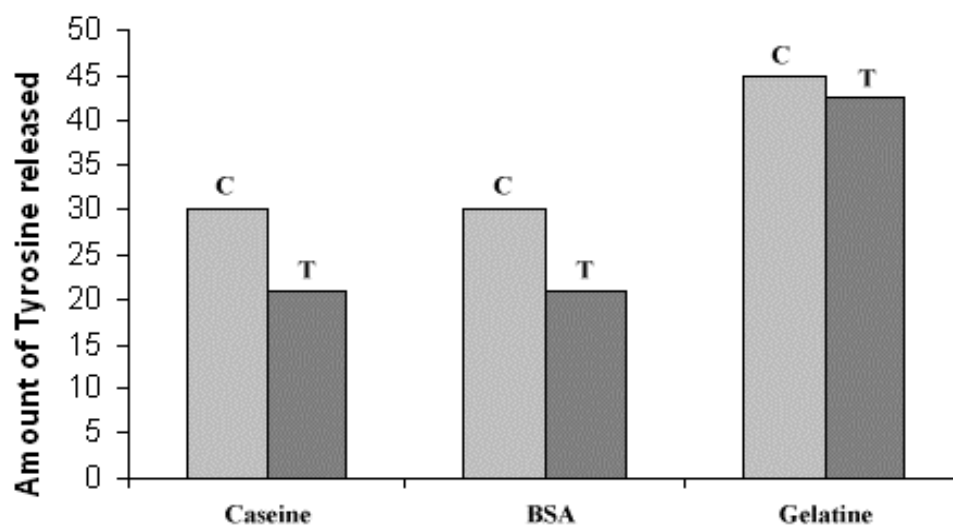
**Figure 3.** Effect of heat shock on protease activity in fifth instar larvae of *Bombyx mori* in presence of different substrates, gelatin (A), casein (B) and BSA (C). C - Protein sample from non-heat shock larvae; HS - Protein sample of silkworm larvae subjected to heat shock at 40°C for 2 h followed by 2 h recovery. Here, protein sample devoid of protease inhibitor was not boiled prior to electrophoresis.

heat shock on differential inhibition of protease activity. Correspondingly, the protease assay performed for the protein samples derived from heat shock induced larvae

**Table 3.** Altered protease activity on different substrates due to heat shock in the larvae of *Bombyx mori*.

Proteolytic bands	Mol. Wt. (kDa)	Gelatin		Casein		BSA	
		C	HS	C	HS	C	HS
P1	116	+	+	-	-	-	-
P2	83	+	+	-	-	-	-
P3	57	+	-	-	-	-	-
P4	35	+	-	-	-	-	-
P5	24	+	-	+	±	+	±

(C- control; HS- heat shock; '+' strong activity; '±' weak activity; '-' no activity).

**Figure 4.** Altered protease activity (µg/ml) in the presence of different substrates (gelatin, casein and BSA) due to heat shock in *Bombyx mori*. Protein sample derived from non-heat shock larvae (C) and larvae subjected to heat shock (T) at 40°C for 2 h followed by 2 h recovery.

revealed decreased protease activity compared to that of control protein sample in all the substrates tested (Figure 4), which is in conformity with that of zymography analysis. Interestingly, the total arginine and lysine-specific protease activities of whole cultures and cell-free supernatant of *Porphyromonas gingival* decreased as the growth temperature increased. Thus, *P. gingivalis* responds to an increased temperature by adopting a less inflammatory and aggressive phenotype while retaining its population (Rimondia et al., 1999). With these inferences, we suppose that a reduction in most active protease P3 and others, no alteration in P1 and P2 activity due to heat shock might have significant role in acclimation to elevated temperature that silkworm *B. mori* experience during rearing in the field conditions. Although, its interaction with the expression of different sets of HSPs in *B. mori* is enigmatic (Aparna et al., 2010; Vasudha et al., 2006), but protein complexes consists of HSPs and other proteins are one of the most common products derived from protein interactions. Evidently,

while in *Thermoanaerobacter brockii* cells exposed to temperature increases from 60 to 65°C, three enzymes (malate dehydrogenase, isocitrate dehydrogenase and alcohol dehydrogenase) were protected from aggregation through interaction HSP60 and HSP10, which were active in protein folding assays (Truscott et al., 1994). Further, several proteins including sHSP detected in most temperature-dependent complexes in *T. tengcongensis* could strengthen the interaction of the bacterial proteins to prevent protein aggregation and degradation (Meng et al., 2009). Thus, it is worth noting that formation of protein complexes at high temperature is not only for avoiding aggregation but also for maintaining protein activities and native structures as have been observed in the present investigation.

## Conclusion

Application of zymography facilitated detection of four

active serine and one unknown proteases in fifth instar larvae, which is the most active feeding and silk protein synthesis stage of the *B. mori*. The differential activity of the proteases both in normal and heat shock induced larvae revealed the possible modification due to imposed thermal stress in the silkworm larvae. Although, detailed studies are required to correlate its role in thermotolerance and adaptation to heat in the domesticated silkworm, *B. mori* also has other naturally growing insects in the context of global warming that affect insect population dynamics.

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